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Gli3 is required autonomously for dorsal telencephalic cells to adopt appropriate fates during embryonic forebrain development

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ABSTRACT

The Gli3 zinc finger transcription factor is expressed in developing forebrain, with the highest levels of expression in dorsal telencephalon. In *Gli3*$^{-/-}$ embryos the dorsal telencephalon is abnormally small and fails to develop dorsomedial telencephalic structures, including hippocampus and cortical hem, while the ventral telencephalon appears to expand. A hurdle to understanding the underlying mechanisms is that abnormalities of developing *Gli3*$^{-/-}$ telencephalic cells in *Gli3*$^{-/-}$ mutants result from a combination of their own cell autonomous defects and defects in the *Gli3*$^{-/-}$ cells that surround them. Here we used chimeras to identify some of the defects of *Gli3*$^{-/-}$ telencephalic cells that are likely to be autonomous by studying how *Gli3*$^{-/-}$ cells develop when surrounded by a majority of wild-type cells. We found that *Gli3*$^{-/-}$ cells are present in all components of the *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeric forebrain, including dorsomedial structures, in proportions that either equal or exceed proportions found elsewhere in the embryo. *Gli3*$^{-/-}$ cells segregate from *Gli3*$^{+/+}$ cells to form many abnormal structures particularly in dorsal telencephalon. *Gli3*$^{-/-}$ cells in some locations are misspecified: in those parts of the dorsal telencephalon near to its boundaries with the diencephalon and the ventral telencephalon, mutant cells express sets of transcription factors expressed by wild-type cells on the other side of the boundary. Elsewhere in the dorsal telencephalon, in the diencephalon and in the ventral telencephalon, mutant cells express sets of transcription factors similar to those expressed by their immediately surrounding wild-type cells. We propose that an important cell autonomous action of Gli3 is to regulate the competence of dorsal telencephalic cells, preventing cells near to its boundaries expressing regulatory factors normally restricted to adjacent tissues.

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Introduction

Gli3 is a zinc finger transcription factor expressed in developing neural and non-neural tissues. It is an important component of the Sonic hedgehog (Shh) signalling pathway and Gli3 protein is cleaved into a short repressor form (Gli3R) in the absence of Shh (Blaess et al., 2006; Meyer and Roelink, 2003). Levels of Gli3R are higher in dorsal structures most distant from the ventral Shh producing regions (Fotaki et al., 2006). It has been suggested that Gli3R plays an active role in specifying dorsal telencephalic tissues (Rash and Grove, 2007), but how it plays such a role remains unclear.

Evidence that Gli3 has important functions in embryogenesis has come from mutant mice lacking Gli3 function. Complete loss of Gli3 causes embryos to die around birth with severe defects at multiple sites including the limbs, which develop with extra digits, and the brain (Hui and Joyner, 1993; Johnson, 1967; Maynard et al., 2002; Theil, 2005; Vortkamp et al., 1992; Winter and Huson, 1988). In the brain of *Gli3*$^{-/-}$ embryos the most obvious defects are in the telencephalon which is severely diminished in size and often exhibits exencephaly (Copp, 1994). *Gli3*$^{-/-}$ brains that are not exencephalic fail to develop dorsomedial telencephalic structures including the hippocampus and cortical hem (Grove et al., 1998; Rash and Grove, 2007; Tole et al., 2000). In the telencephalon, only a small remnant of dorsal telencephalic tissue persists between diencephalon and ventral telencephalon. This residual neocortex contains groups of cells that express diencephalic markers surrounded by dorsal telencephalic cells (Fotaki et al., 2006).

The explanation for the findings in *Gli3*$^{-/-}$ embryos is likely to be complex. It is known that loss of Gli3 results in changes in the expression of key signalling molecules arising from signalling centres around the midline that would normally affect the development of the dorsal telencephalon. In the *Gli3*$^{-/-}$ mutant forebrain, Fgf8 expression is expanded rostrally (Aoto et al., 2002; Theil et al., 1999) while Wnt expression in the dorsomedial telencephalon is greatly reduced (Grove et al., 1998; Kuschel et al., 2003; Tole et al., 2000). It is likely that these changes in the production of signalling molecules affect the development of dorsal telencephalic structures, but they might not provide a full explanation. It is also possible that loss of Gli3 renders dorsal telencephalic cells intrinsically incompetent to generate and/or

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maintain certain dorsal telencephalic tissues, either by preventing dorsal telencephalic cells proliferating and/or surviving in these regions or by causing them to adopt inappropriate fates. Testing this possibility requires approaches additional to the analysis of *Gli3*$^{-/-}$ embryos, in which the phenotype is a net result of both cell non-autonomous and cell autonomous defects.

Here, we studied the development of *Gli3*$^{-/-}$ cells in the forebrain of chimeric embryos containing a majority of wild type cells (designated *Gli3*$^{+/+}$↔*Gli3*$^{-/-}$ or *Gli3*$^{-/-}$ chimeras). The principle of this approach is that defects retained by *Gli3*$^{-/-}$ cells are likely to be cell autonomous. We studied chimeras in which proportions wild-type cells were relatively high, so as to optimize their ability to generate forebrain tissues regardless of the presence of mutant cells in their midst. We found that, despite the presence of mutant cells, wild-type cells did create dorsal telencephalic structures that are missing in *Gli3*$^{-/-}$ embryos. This allowed us to ask about the contributions and gene expression patterns of mutant cells embedded in an environment of wild-type cells in the process of creating all of the main forebrain structures. Our results indicate that *Gli3*$^{-/-}$ cells do contribute to these structures but that do not attain their normal identities: many *Gli3*$^{-/-}$ cells in dorsal telencephalon attain characteristics of either ventral telencephalon or diencephalon, depending on their location.

Materials and methods

Derivation of Gli3$^{-/-}$ *embryonic stem (ES) cells*

We used mice carrying the *Xt*^{J} null allele, a large deletion encompassing much of the *Gli3* gene (Vortkamp et al., 1992). *Gli3*$^{+/+}$ or *Gli3*$^{-/-}$ ES cells (129Sv background) were derived from embryos obtained from *Gli3*$^{+/-}$ female mice that were superovulated and mated with male *Gli3*$^{+/-}$ mice that were in some cases homozygous for a reiterated β-globin repeat transgene ([TgN(Hbb-bl)83Clo] — abbreviated to *Tg* hereafter) (Lo et al., 1987). ES cells were derived from individually cultured blastocysts (Turksen, 2002). The resulting ES cell lines were then passaged in feeder-free conditions in BHK-21 Glasgow MEM (GMEM; Invitrogen) with 15% fetal bovine serum (FBS) and LIF (1000 U/ml). Cell lines were genotyped for the *Xt*^{J} allele and the *Tg* transgene and their glucose phosphate isomerase (GPI) isotypes were determined, as described previously (Quinn et al., 2007; Zaki et al., 2006). All ES cell lines generated were karyotyped and those used for chimera generation had a normal chromosome complement.

Labelling ES cells by in vitro transfection

Subconfluent *Gli3*$^{+/+}$ or *Gli3*$^{-/-}$ ES cells were dissociated using Tryple Express™ (Invitrogen, UK) and plated in GMEM medium containing LIF and 15% FCS. Twenty four hours later, Fugene (Invitrogen, UK) was used to transfect cells with a plasmid (pEGFPN1; Clontech, UK) that expresses enhanced green fluorescent protein (EGFP) in all cells. Transfected cells were selected in medium containing 600 μg/μl G418. After 7 days in selection medium individual colonies were picked and expanded by passage in GMEM+FBS with LIF (1000 U/ml). Stably transfected EGFP expressing lines were used for blastocyst injections.

Chimera production and analysis

Experimental *Gli3*$^{+/+}$↔*Gli3*$^{-/-}$ and control *Gli3*$^{+/+}$↔*Gli3*$^{+/+}$ chimeras were produced by three methods. (1) Injection of *Gli3*$^{-/-}$ ES cells into *Gli3*$^{+/+}$ blastocysts that were hemizygous for *Tg*. (2) Injection of wild type ES cells into blastocysts that were *Gli3*$^{-/-}$;*Tg*, or *Gli3*$^{+/-}$;*Tg*, or *Gli3*$^{+/+}$;*Tg* (from *Gli3*$^{+/-}$; *TgTg*×*Gli3*$^{+/-}$ crosses). (3) Injection of *Gli3*$^{-/-}$; *EGFP* or *Gli3*$^{+/+}$; *EGFP* ES cells into wild type blastocysts. Blastocysts were placed into recipient females which were killed at several subsequent ages. Chimeric embryos were genotyped according to Zaki et al. (2006). In the case of chimeras made by methods (1) and (3) tissue was taken from the trunk of the embryo for genotyping while for chimeras produced by method (2) the yolk sac endoderm (which is not colonized by ES cells: Tam and Rossant, 2003) was dissected away from other extra-embryonic tissues and used for determining the genotype of the original host blastocysts (West and Flockhart, 1994). The trunk of each embryo was used to obtain a measure of the global contribution of ES cell-derived embryonic tissue using GPI electrophoresis as described previously (Quinn et al., 2007). ES cell lines were always GPI1A and donor blastocysts GPI1B. Embryos were fixed in 4% paraformaldehyde, wax-embedded and sections were cut (10 μm). Where the *Tg* marker was included, it was revealed by DNA-DNA in situ hybridisation (Lo et al., 1987; Quinn et al., 2007). Counts of *Tg*-positive and *Tg*-negative cells were obtained using StereoInvestigator™ (MBF Bioscience, USA). The programme randomly assigned 150×150 μm counting boxes within each of a series of normal or abnormal forebrain tissues delineated as described in Results.

Immunohistochemistry

Antibodies to the following were used on wax-embedded sections using conventional methods: FoxG1 (1:100; Abcam, UK), calretinin (1:400; Swant Antibodies, Switzerland), reelin (1:100; Chemicon, UK), Nkx2.2 (1:100; Developmental Studies Hybridoma Bank [DSHB], USA), Pax6 (1:100; DSHB, USA), Mash1 (1:100; BD Biosciences, UK), Tbr2 (1:200; gift from R. Hevner, University of Washington, Seattle, USA), phosphohistone-3 (pH3; 1:400; Sigma, UK), Lim1/2 (1:200; DSHB, USA), Gsh2 (1:2500; gift from K. Campbell, Children's Hospital Research Foundation, Cincinnati, USA), MAP2 (1:100; Sigma, UK), Gad67 (1:100; Chemicon, UK), Nkx2.1 (1:100; Developmental Studies Hybridoma Bank [DSHB], USA), and Dlx2 (Abcam, UK, 1:100). Visualisation was achieved using anti-mouse or anti-rabbit biotin-conjugated secondary antibodies, amplified (ABC kit, Vector Laboratories, UK) and visualised by staining with diaminobenzidine (DAB) (Invitrogen, UK). In situ hybridisation for the presence of the β-globin transgene in conjunction with immunohistochemistry was performed as described previously (Quinn et al., 2007), and was possible for many of the antibodies (see Results). In some cases where we were unable to get antibodies to stain on the same section as used for in situ hybridisation, we compared adjacent sections reacted immunohistochemically and with in situ hybridisation to identify clusters of *Gli3*$^{-/-}$ cells (see Results).

Results

Chimera production

In total, 12 *Gli3*$^{+/+}$↔*Gli3*$^{-/-}$ chimeras and 15 *Gli3*$^{+/+}$↔*Gli3*$^{+/+}$ chimeras were generated at embryonic days (E) 11.0, 12.5, 14.5 and 15.5 with global contributions of *Gli3*$^{-/-}$ cells (estimated by GPI1 isotype analysis, see Materials and methods and Quinn et al., 2007) ranging from 19% to 38%. *Gli3*$^{+/+}$↔*Gli3*$^{-/-}$ chimeras displayed 5–7 digits on each of their limbs, with the extra digits being located preaxially, postaxially and/or within the planar digits of the forelimb or hindlimb. *Gli3*$^{-/-}$ embryos on the background strain used here to generate ES cells usually have either 7 or 8 digits of uniform size on each of their forelimbs and hindlimbs. In chimeras, the digital anomaly was diagnostic for *Gli3*$^{+/+}$↔*Gli3*$^{-/-}$ genotype, but apart from these limb defects the external appearance of *Gli3*$^{+/+}$↔*Gli3*$^{-/-}$ chimeras was indistinguishable from that of *Gli3*$^{+/+}$↔*Gli3*$^{+/+}$ and wild type mice.

The forebrains of Gli3$^{+/+}$↔*Gli3*$^{-/-}$ *chimeras show major morphological defects*

The most obvious defect of non-chimeric *Gli3*$^{-/-}$ telencephalon is failure of dorsal midline invagination and absence of dorsomedial telencephalic structures including the hippocampus and cortical hem

(Grove et al., 1998; Theil et al., 1999; Tole et al., 2000). In $Gli3^{+/+} \leftrightarrow Gli3^{-/-}$ chimeras dorsal midline invagination and structures resembling hippocampus and cortical hem were observed (Figs. 1A–F).

In all $Gli3^{+/+} \leftrightarrow Gli3^{-/-}$ chimeras, ventral telencephalon and diencephalon appeared relatively normal but dorsal structures contained numerous abnormalities (Fig. 1). These abnormalities were consistent between $Gli3^{+/+} \leftrightarrow Gli3^{-/-}$ chimeras. Some parts of the neocortex were thicker than normal and there were large clusters of cells (marked with asterisks in Fig. 1B), some of which contained a central lumen. These clusters distorted the shape of the neocortex, including those regions with normal thickness and appearance (e.g. in the right hemisphere in Fig. 1B). Clusters of cells were observed in three locations: (1) in the ventral pallium near to the pallial/subpallial boundary (PSPB) (marked lat* in Fig. 1B); (2) throughout the dorsal to lateral extent of the cortical plate (e.g. indicated by asterisks in Figs. 1B and D); (3) medial to the hippocampus (indicated as med* in Fig. 1F). In addition, ectopic tissue was seen bilaterally between the cortical hem and the choroid plexus (indicated as # in Fig. 1B).

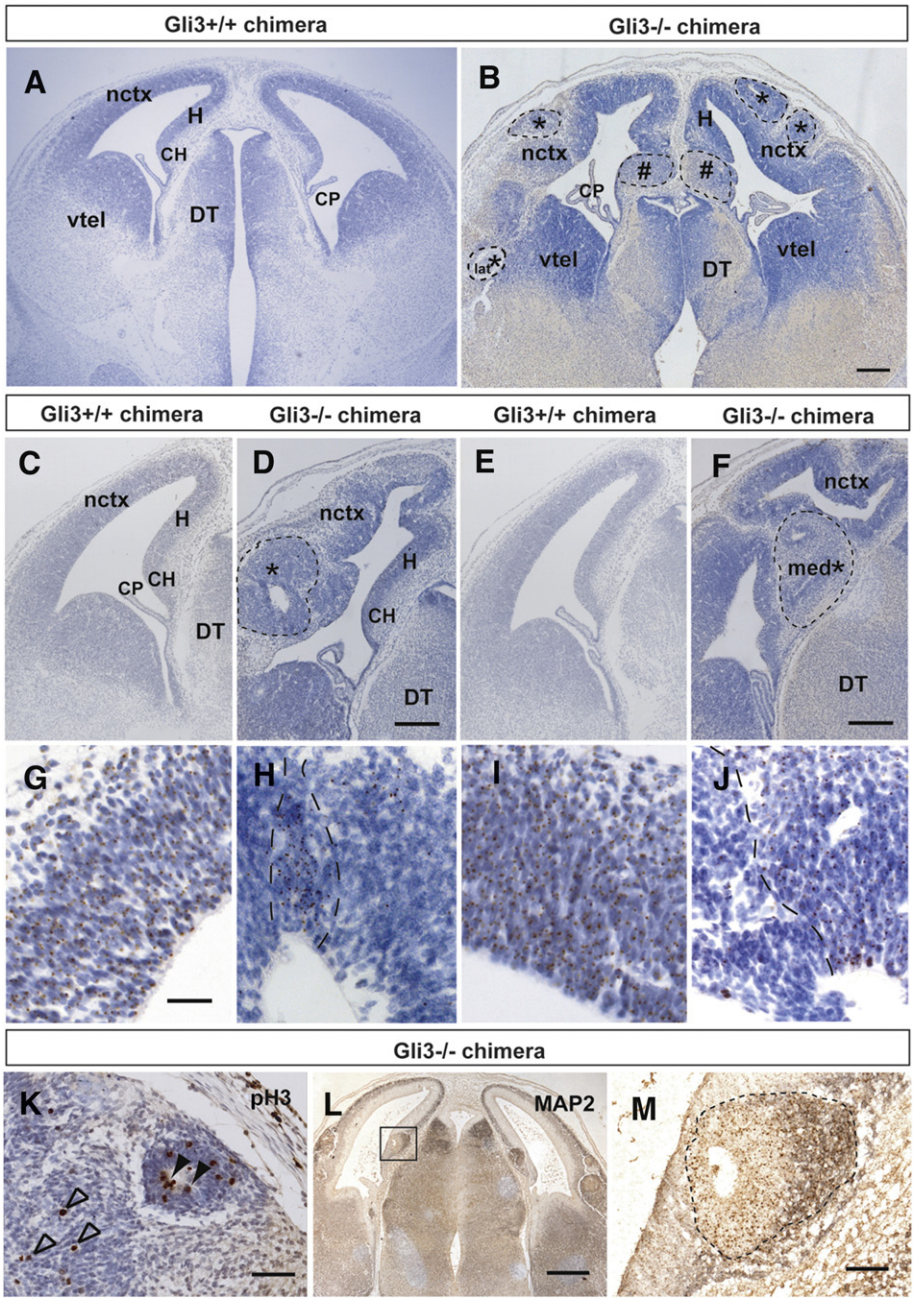

Fig. 1. Telencephalic dorsal midline invaginates but forebrain development is abnormal in $Gli3^{-/-} \leftrightarrow Gli3^{+/+}$ chimeras. (A) Section of an E14.5 $Gli3^{+/+} \leftrightarrow Gli3^{+/+}$ chimera showing normal forebrain morphology. (B) Section at a comparable level to that in (A) of an E14.5 $Gli3^{-/-} \leftrightarrow Gli3^{+/+}$ chimera containing 24.5% $Gli3^{-/-}$ cells. Major morphological defects, seen in all $Gli3^{-/-} \leftrightarrow Gli3^{+/+}$ chimeras of similar age, are in the dorsal telencephalon. They include thickening the cortex, numerous abnormal clusters of cells (*), some of which are situated near to the PSPB (lat*), and extra tissue dorsal to the choroid plexus (#). (C–F) Sections from the chimeras represented in (A, B) taken further caudally. (G–J) Sections through the neocortex (G, H) and hippocampus (I, J) of chimeras in which $Gli3^{+/+}$ cells carry the *Tg* marker, revealed as a brown dot in the nucleus. (G, I) In control chimeras *Tg*-positive and *Tg*-negative cells intermingle; (H, J) in experimental chimeras there is segregation between $Gli3^{-/-}$ and $Gli3^{+/+}$ cells (broken lines outline zones of segregation). (K) Phosphohistone-H3 (pH3) staining of cells in the proliferative zone (open arrowheads) and abnormal clusters (filled arrowheads) in the cortex of an experimental chimera. (L, M) MAP2 staining of the forebrain of an experimental chimera showing that the abnormal clusters, containing $Gli3^{-/-}$ cells (which are in this case *Tg*-positive), contain differentiated neurons (M is higher magnification of boxed area in L). Abbreviations: nctx, neocortex, vtel, ventral telencephalon, H, hippocampus, CH, cortical hem; CP, choroid plexus; DT dorsal thalamus; med*, medial dorsal telencephalic cluster; lat* lateral dorsal telencephalic cluster. Scale bars: A, L=400 μm; B–F=200 μm; G–K,M=50 μm.

In *Gli3*$^{+/+}$↔*Gli3*$^{+/+}$ control chimeras, *Tg*-positive and *Tg*-negative cells intermingled in all tissues (illustrated in Figs. 1G,I). In *Gli3*$^{+/+}$↔*Gli3*$^{-/-}$ experimental chimeras, *Gli3*$^{-/-}$ cells were present throughout forebrain tissues including those with relatively normal appearance, but were segregated, particularly in dorsal telencephalon. Figs. 1H,J illustrate this separation of wild type *Tg*-positive cells (marked by brown dots in their nuclei) from *Gli3*$^{-/-}$ *Tg*-negative cells in cortex of relatively normal thickness. The abnormal clusters of cells designated by asterisks and # in Figs. 1B,D,F contained very high proportions of *Gli3*$^{-/-}$ cells (*Tg*-positive cells in Fig. 1M). Quantitative data on the contributions of mutant and wild type cells to forebrain structures in chimeras are provided below.

We examined the organization of dorsal telencephalic clusters of *Gli3*$^{-/-}$ cells in more detail by staining for their expression of phosphohistone-H3 (pH3), a marker of mitotic cells, and microtubule associated protein 2 (MAP2), a marker of differentiating neurons (Figs. 1K–M). In wild type cortex, pH3 is expressed by dividing radial glial cells at the ventricular surface and intermediate progenitors within the subventricular zone (e.g. open arrowheads in Fig. 1K) (Quinn et al., 2007). Many pH3-positive cells were observed in the clusters, particularly towards their interior (filled arrowheads in Fig. 1K). MAP2, which is normally expressed in neurons of the cortical plate, was expressed around the edge of the clusters (Figs. 1L, M). These data indicate that segregated clusters of mutant cells comprised both proliferative and differentiating populations. We compared the densities of pH3-positive cells in the neocortical clusters of *Gli3*$^{-/-}$ cells to those in nearby regions of cortex comprising almost exclusively *Gli3*$^{+/+}$ cells at E14.5. Densities were significantly higher in the clusters of mutant cells (*Gli3*$^{-/-}$ clusters: mean 6.1±0.88 s.e.m. cells 0.01 mm^{-2}; *Gli3*$^{+/+}$ cortex: mean 3.4± 0.17 s.e.m. cells 0.01 mm^{-2}; $p<0.002$, Student's *t*-test). This increased density of pH3-postive cells was not explained by an overall increase in cell density in the *Gli3*$^{-/-}$ clusters (*Gli3*$^{-/-}$ clusters: 430.6±19.15 cells 0.01 mm^{-2}; *Gli3*$^{+/+}$ cortex: 473.4±15.95 cells 0.01 mm^{-2}), indicating that there was an increased proportion of dividing cells in the *Gli3*$^{-/-}$ clusters.

Gli3$^{-/-}$ cells segregate from *Gli3*$^{+/+}$ cells in a region-specific manner in *Gli3*$^{+/+}$↔*Gli3*$^{-/-}$ chimeric forebrain

We determined the proportions of (i) *Tg*-negative *Gli3*$^{-/-}$ cells in *Gli3*$^{+/+}$↔*Gli3*$^{-/-}$ E14.5 experimental chimeras and (ii) *Tg*-negative *Gli3*$^{+/+}$ cells in *Gli3*$^{+/+}$↔*Gli3*$^{+/+}$ E14.5 control chimeras in a series of regions through the forebrain. We examined the contribution of these cells to each major diencephalic or telencephalic region. Results were highly consistent between chimeras of similar genotype: results from four chimeras are shown in Fig. 2.

The following regions in control chimeras and their clear equivalents in experimental chimeras were analysed: (i) dorsal thalamus (DT), (ii) eminentia thalami (ET), (iii) hippocampus (H), (iv) neocortex (Nctx), (v) lateral ganglionic eminence (LGE), (vi) medial ganglionic eminence (MGE) and (vii) caudal ganglionic eminence (CGE). The following additional tissues were analysed in *Gli3*$^{+/+}$↔*Gli3*$^{-/-}$ chimeras: (i) tissue lying between the cortical hem and the choroid plexus (indicated by # in Figs. 1B and 2B), (ii) clusters medial to the hippocampus (indicated as med* in Figs. 1F and 2B); (iii) parts of the neocortex that were abnormally thickened (designated nctx abnorm in Fig. 2B); (iv) neocortical clusters (indicated as * in Fig. 1B and nctx* Fig. 2B); (v) clusters in the ventral pallium close to the PSPB (indicated as lat* in Fig. 1D and Fig. 2B).

In control chimeras (Fig. 2A), the contributions of *Tg*-negative cells in each region were very close to the contributions predicted by GPI analysis (red lines in Fig. 2A). In experimental chimeras (Fig. 2B), extremely high percentages of *Gli3*$^{-/-}$ cells (~80–100%) were found in structures with no clear counterparts in control chimeras (designated #, med*, lat*, nctx*). In some cases, these abnormal structures contained pockets of *Gli3*$^{+/+}$ cells that were highly segregated from the *Gli3*$^{-/-}$ cells: this segregation underlies the large variation in the percentages recorded in different sampling boxes from the neocortical clusters in experimental chimera 2 (Fig. 2B, nctx*), since some boxes contained all mutant cells while a few contained mainly wild type cells. There were also large variations in counts from many of the regions of experimental chimeras that were recognizably the

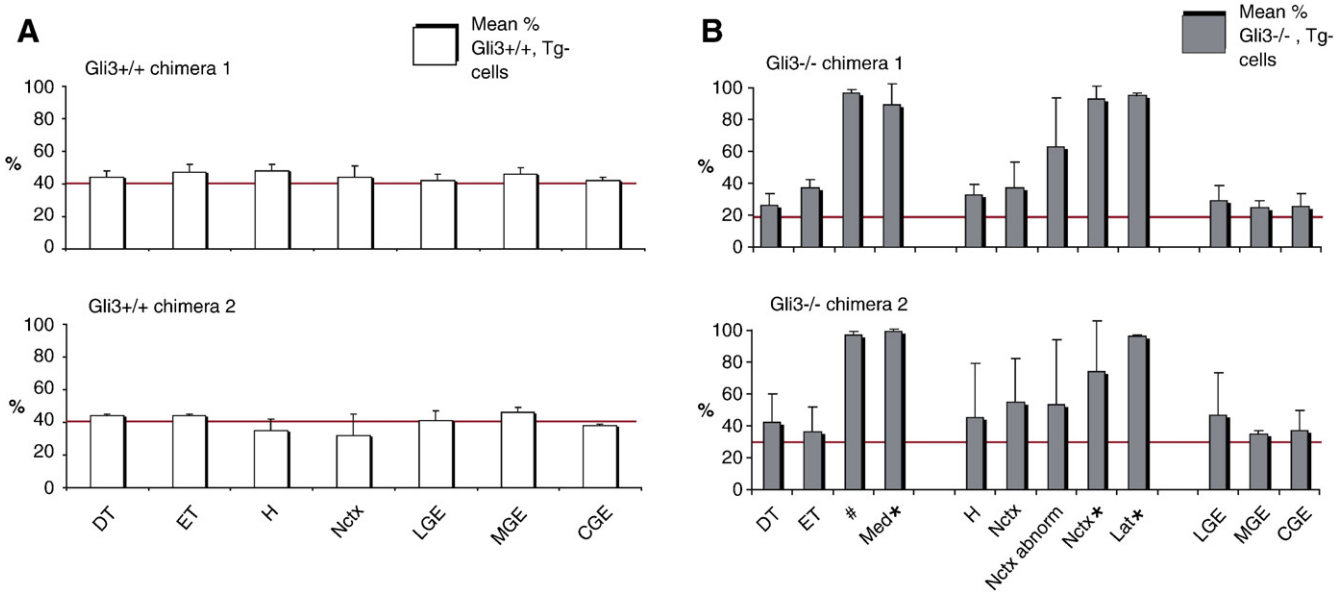

Fig. 2. *Gli3*$^{-/-}$ cells contribute to forebrain tissues in proportions that are similar to or greater than those elsewhere in *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeras. (A) In control chimeras the % contribution of *Tg*-negative *Gli3*$^{+/+}$ cells is close to that predicted by GPI1 analysis (red line). (B) In experimental *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeras the % contribution of *Tg*-negative *Gli3*$^{-/-}$ cells is close to that predicted by GPI1 analysis (red line) in diencephalic (DT and ET) and ventral telencephalic regions (LGE, MGE and CGE), whereas other structures had higher than expected contributions of *Gli3*$^{-/-}$ cells, in some cases up to almost 100%. Values are means (±s.e.m.) of proportions from a series of counting boxes in each area. Note that, in each region, there is greater variation between values from each counting box in experimental chimeras, reflecting the segregation of cells with different *Gli3* genotypes. Abbreviations: ET, eminentia thalami; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; CGE, caudal ganglionic eminence; Nctx abnorm, neocortex with abnormal thickness; Nctx*, neocortical clusters; others as in Fig. 1.

counterparts of wild type structures (Fig. 2B), particularly those located more dorsally, reflecting the underlying segregation of cells with different genotypes.

In the forebrain of the youngest experimental chimeras, aged E11.0, there was already clear evidence of the segregation of *Gli3*$^{+/+}$ and *Gli3*$^{-/-}$ cells apparent in older chimeras. Fig. 3 shows examples of control chimeras (Figs. 3A,B) and experimental chimeras (Figs. 3C–F) in which the ES-derived *Gli3*$^{+/+}$ cells (in controls) or *Gli3*$^{-/-}$ cells (in experimental embryos) express GFP and are *Tg*-positive. Whereas in *Gli3*$^{+/+}$↔*Gli3*$^{+/+}$ chimeras the GFP-positive cells mixed evenly with the GFP-negative cells (Figs. 3A,B), in *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeras there was obvious clustering of *Gli3*$^{-/-}$ cells in the dorsal telencephalon and at the junction between telencephalon and diencephalon (Figs. 3C,D,F). Segregation of *Gli3*$^{-/-}$; *Tg*; *GFP* cells was not apparent in sections of the ventral telencephalon of E11.0 *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeras (Fig. 3E).

In summary, *Gli3*$^{-/-}$ cells segregate from *Gli3*$^{+/+}$ cells in the chimeric forebrain to an extent that varies with region. The greatest segregation occurs in the dorsal telencephalon and in the vicinity of the telencephalic/diencephalic boundary; highly abnormal structures are created in these regions. Segregation of *Gli3*$^{+/+}$ and *Gli3*$^{-/-}$ cells is less extreme in ventral telencephalon and diencephalon.

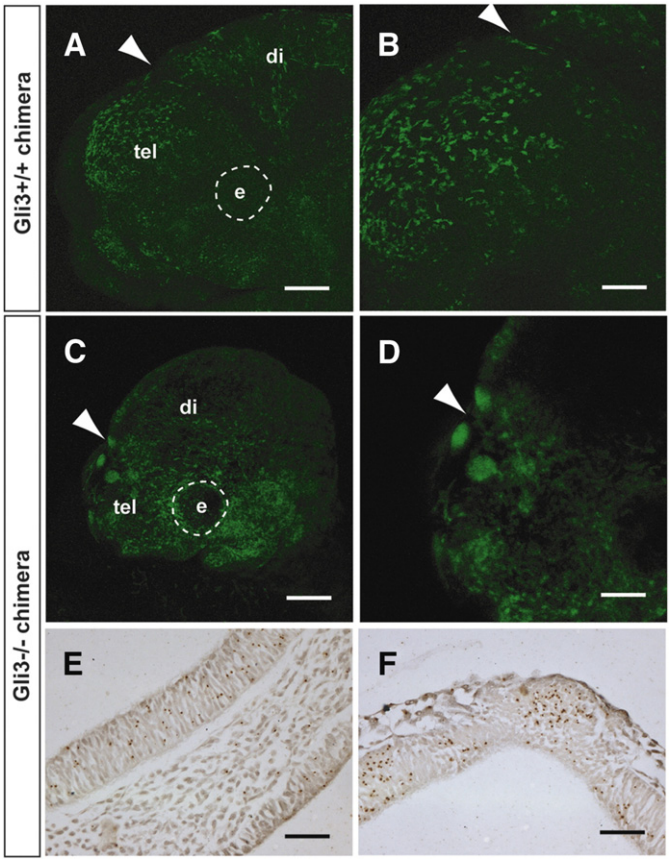

Fig. 3. Segregation of *Gli3*$^{-/-}$ and *Gli3*$^{+/+}$ cells in the dorsal telencephalon of E11.0 *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeras. (A, B) Whole-mount control chimeras in which one of the two sets of *Gli3*$^{+/+}$ cells (making a 25% contribution) expresses EGFP. EGFP-positive cells were dispersed throughout the forebrain (arrowhead indicates the telencephalic/diencephalic boundary). (C, D) Whole-mount experimental chimeras in which *Gli3*$^{-/-}$ cells (making a 20% contribution) express EGFP: EGFP-positive cells were clustered in the dorsal telencephalon, particularly in the vicinity of the telencephalic/diencephalic boundary (arrowhead). (E,F) DNA-DNA in situ hybridisation for the β-globin transgene (*Tg*; revealed as brown nuclear dots) in sections of a *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimera showing clustering in (F) dorsal telencephalon but not (E) ventral telencephalon. Abbreviations: tel, telencephalon; di, diencephalon; e, eye. Scale bars: A, C, 400 μm; B, D, 100 μm; E, F, 50 μm.

Gli3$^{-/-}$ *cells in the Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ *chimeric telencephalon exhibit altered identity in a location-specific manner*

A possible reason for segregation of mutant cells is a fundamental alteration in their identity. We tested this possibility using a range of markers expressed selectively in specific regions of the forebrain.

Foxg1 expression

In normal embryos, the forkhead transcription factor Foxg1 is expressed throughout the telencephalon but not the diencephalon (Tao and Lai, 1992; Xuan et al., 1995). In *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeras (E14.5, *n*=3), Foxg1 expression was absent from both *Gli3*$^{-/-}$ and *Gli3*$^{+/+}$ cells in diencephalon (Figs. 4A,B,J) and was present in both *Gli3*$^{-/-}$ and *Gli3*$^{+/+}$ cells throughout much of the telencephalon, with the exception of *Gli3*$^{-/-}$ cells located dorsomedially (Figs. 4A–I). At caudal levels (Fig. 4A), *Gli3*$^{-/-}$ cells situated dorsally (Fig. 4D) and medially (Fig. 4G) did not express Foxg1. Further rostrally (Fig. 4B), medially located *Gli3*$^{-/-}$ cells including those in the medial structure designated # did not express Foxg1 (Fig. 4H). At rostral levels (Fig. 4C), stripes of *Gli3*$^{-/-}$ cells in the medial dorsal telencephalic wall showed either no or only very faint staining for Foxg1 (Fig. 4F). At caudal and central levels, *Gli3*$^{-/-}$ cells located further laterally, in the neocortex, showed staining for Foxg1 at similar levels to their wild type neighbours (Fig. 4E). Rostrally, while *Gli3*$^{-/-}$ cells situated laterally in the neocortex were clearly positive for Foxg1, they were stained less strongly that their wild type neighbours (Fig. 4I). In the ventral telencephalon, the intensity of staining for Foxg1 was similar between *Gli3*$^{+/+}$ and *Gli3*$^{-/-}$ cells (Fig. 4K,L).

In summary, *Gli3*$^{-/-}$ cells situated in the telencephalon closer to the dorsal midline and the boundary with the diencephalon do not express Foxg1 whereas *Gli3*$^{-/-}$ cells situated further from the dorsal midline and the boundary with the diencephalon do express Foxg1.

Gsh2 expression

Expression of Gsh2 is normally restricted to proliferating cells of the ventral telencephalon and the ventral thalamus (Hsieh-Li et al., 1995). In experimental chimeras (E12.5, *n*=1; E14.5, *n*=3; E15.5, *n*=1), Gsh2 expression also encroached into parts of the dorsal telencephalon, where it was found in (i) many *Gli3*$^{-/-}$ cells near to the dorsal midline and the boundary with the diencephalon (ii) *Gli3*$^{-/-}$ cells in rostral and lateral regions. Figs. 5A,D,G show a caudal section with expression of Gsh2 in the clusters of *Gli3*$^{-/-}$ cells located medial to the developing neocortex (Fig. 5A, boxed area is shown in D, *Gli3*$^{-/-}$ cells were *Tg*-negative and contained no brown nuclear dots); Gsh2 was expressed by both wild-type and mutant cells in ventral telencephalon (Fig. 5G). In central and rostral sections, ectopic Gsh2 expression was present in groups of *Gli3*$^{-/-}$ cells located in the proliferative zone of the medial wall of the dorsal telencephalon (Figs. 5B,C,E,F); surrounding *Gli3*$^{+/+}$ (*Tg*-positive) cells were Gsh2-negative (Figs. 5E, F). Many *Gli3*$^{-/-}$ cells located on the dorsal side of the PSPB expressed Gsh2 ectopically (Figs. 5B,C,H). Apart from at rostral levels, where ectopic expression was throughout the dorsal telencephalon (Fig. 5C), Gsh2 was not expressed by *Gli3*$^{-/-}$ cells located in the central part of the neocortex (Fig. 5B,I). The abnormal *Gli3*$^{-/-}$ region designated # did not express Gsh2 and nor did the eminentia thalami (Fig. 5B; (Hsieh-Li et al., 1995).

In summary, in *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeras mutant cells in the eminentia thalami and the adjacent ectopic tissue designated #, which surround the diencephalic/telencephalic border, express neither Foxg1 nor Gsh2. Moving away from these tissues and further into the telencephalon, *Gli3*$^{-/-}$ cells in segregated stripes and clusters in the medial dorsal telencephalon are Foxg1-negative, Gsh2-positive, a combination normally associated with ventral thalamic cells. Moving yet further into the dorsal telencephalon, *Gli3*$^{-/-}$ cells in the central portion of the neocortex are Foxg1-positive, Gsh2-negative, a combination found in wild-type cells in this location. Approaching the

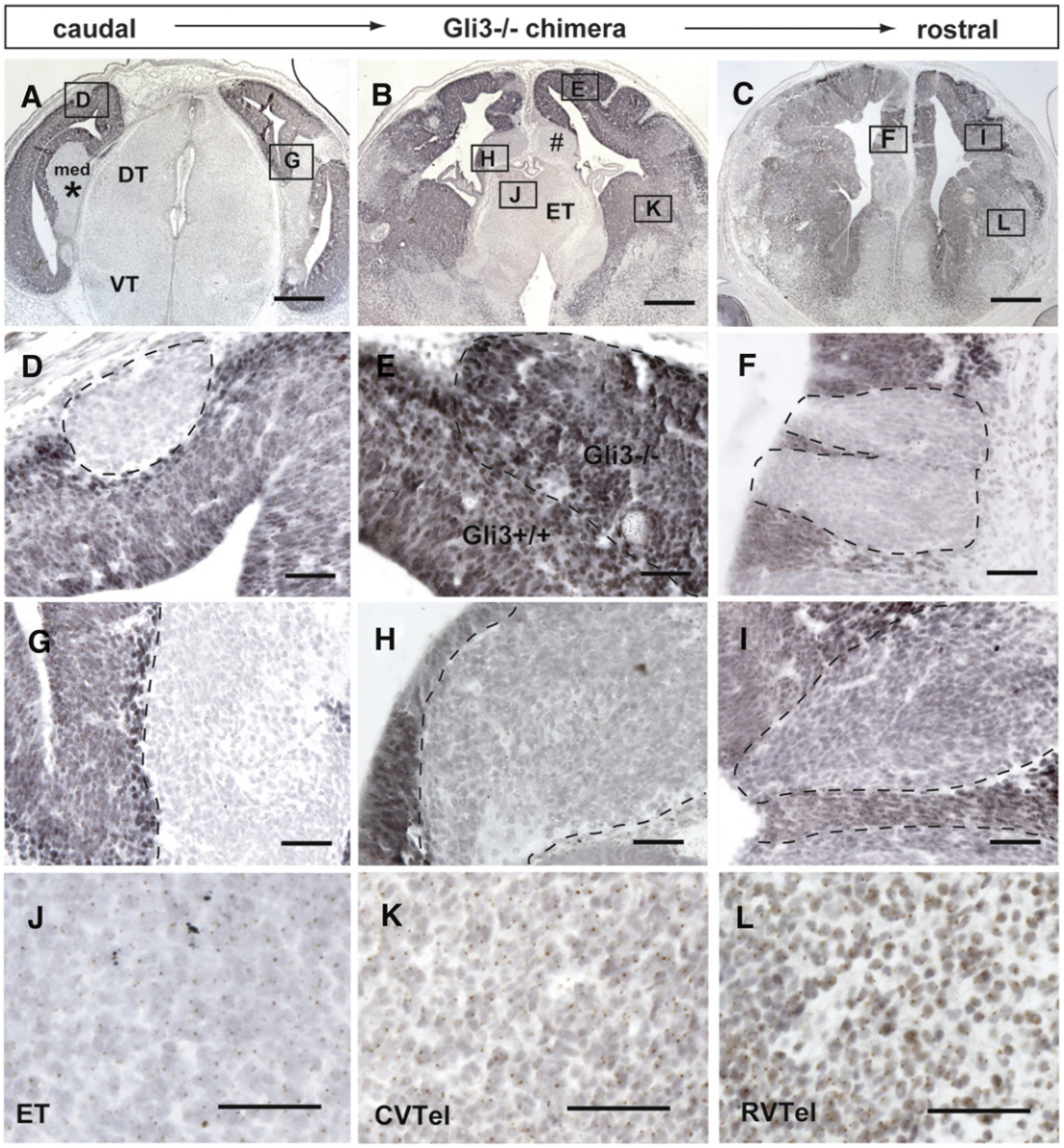

Fig. 4. Foxg1 expression is perturbed in telencephalic *Gli3*$^{-/-}$ cells in *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeras. Immunohistochemistry on E14.5 *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimera in which wild type cells are *Tg*-positive. (A) Caudal section from the forebrain of a *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimera containing 30% *Gli3*$^{-/-}$ cells. Clusters of *Gli3*$^{-/-}$ cells located within the dorsomedial telencephalon (boxed areas shown in D and G) do not express Foxg1. (B) Central section from the same chimera: in the centre of the neocortex both mutant and wild-type cells express Foxg1 (boxed area shown in E), mutant cells in the tissue designated # (Fig. 1B) do not express Foxg1 (boxed area shown in H), neither mutant nor wild type cells in the eminentia thalami express Foxg1 (boxed area shown in J) and both wild type and mutant cells express Foxg1 in ventral telencephalon (boxed area shown in K). (C) Rostral section showing widespread Foxg1 expression throughout the telencephalon, although mutant cells located in the medial telencephalic wall exhibit little or no Foxg1 expression (boxed area shown in F: mutant cells are outlined by a broken line. Wild type and mutant cells elsewhere in the section express Foxg1 (boxed areas shown in I and L). Abbreviations: VT, ventral thalamus; CVTel, central ventral telencephalon; RVTel, rostral ventral telencephalon; others as in Figs. 1 and 2. Scale bars: A–C=400 μm; D–L=50 μm.

rostral and lateral edges of the dorsal telencephalon, *Gli3*$^{-/-}$ cells are Foxg1-positive, Gsh2-positive, a combination normally associated with ventral telencephalon.

Lim1/2 expression

The antibody used here labels differentiating diencephalic cells expressing Lim 1 and/or Lim2 (Lhx1 and/or 5) in the eminentia thalami, ventral thalamus and epithalamus (Sheng et al., 1997; Fotaki et al., 2006). In experimental chimeras (E14.5, n=3; E15.5, n=2), additional expression of Lim1/2 was found in *Gli3*$^{-/-}$ cells in the dorsal and medial telencephalic wall (marked * and med* in Fig. 6A, shown in C and E) and the region designated # (Figs. 6B,D). Lim1/2 expression was detected in neither *Gli3*$^{-/-}$ nor *Gli3*$^{+/+}$ cells in most of the rest of the dorsal telencephalon (Figs. 6B,F; note that staining in panel B marked by arrowhead is an artefact), with the exception of the marginal zone, where it is known to be expressed in Cajal–Retzius cells (Fig. 6F) (Yamazaki et al., 2004). These findings indicate that *Gli3*$^{-/-}$ cells in medial (but not lateral) parts of the dorsal telencephalon have adopted molecular identities associated with ventral thalamus and eminentia thalami. Data in Fig. 6F and Supplementary Fig. 1 indicate no major change in numbers of Reelin-positive Cajal–Retzius cells in *Gli3*$^{-/-}$ neocortical regions in chimeras.

Tbr2 expression

Normally, Tbr2 is expressed in the dorsal telencephalon and the eminentia thalami, but is not expressed in the main body of either the ventral or dorsal thalamus (Brox et al., 2004; Englund et al., 2005; Quinn et al., 2007) (Quinn, unpublished observations). Tbr2 was

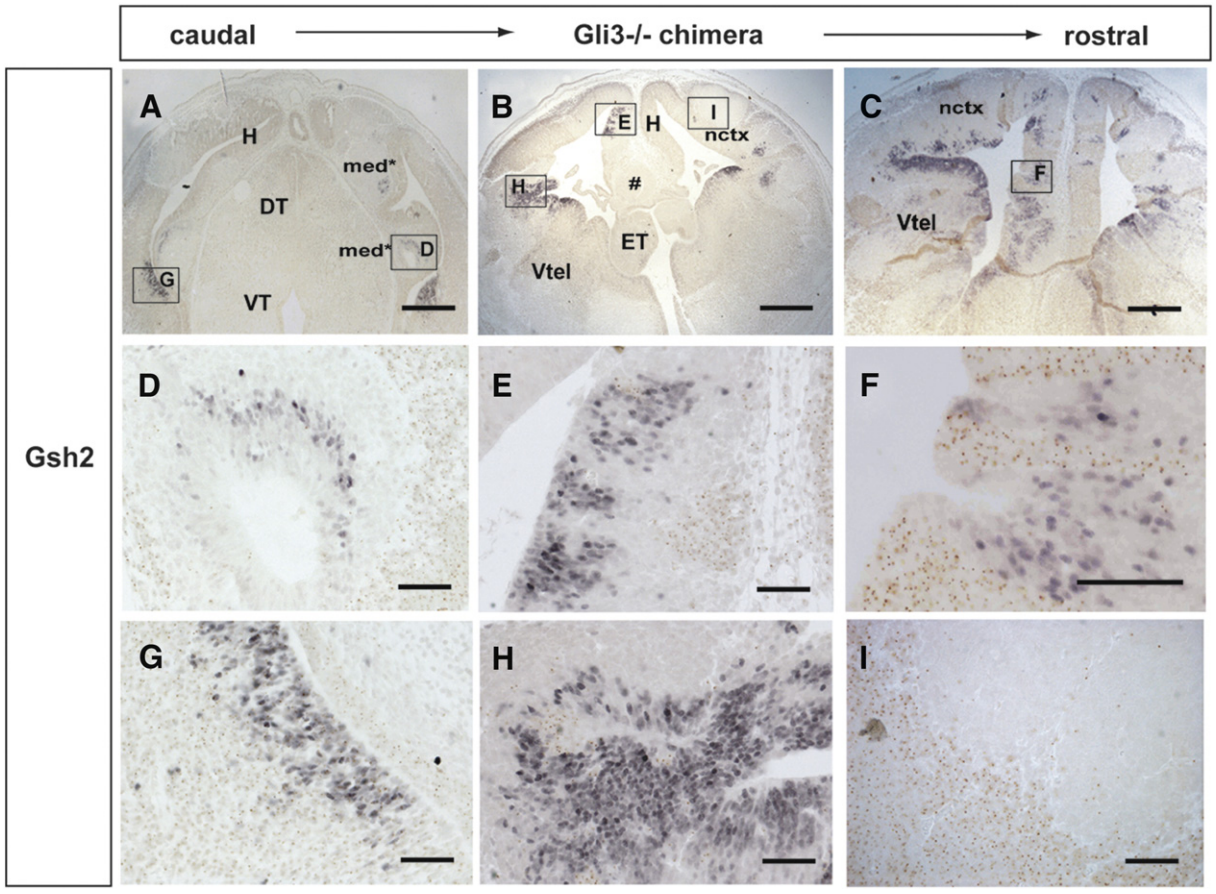

Fig. 5. *Gli3*$^{-/-}$ mutant cells in the forebrain of *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeras inappropriately express the ventral marker Gsh2. Immunohistochemistry on E14.5 *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimera in which wild type cells are *Tg*-positive. (A) In caudal sections, ectopic Gsh2 expression was observed in medial clusters of *Gli3*$^{-/-}$ cells (boxed area shown in D). Gsh2 was expressed in both mutant and wild type cells in ventral telencephalon (G). (B) In central sections, Gsh2 was ectopically expressed in *Gli3*$^{-/-}$ cells in the medial telencephalic wall (boxed area shown in E) and around the angle of the lateral cortex (boxed area shown in H). In contrast, neither the large clusters of mutant cells in the neocortex (boxed area shown in I) nor the structure comprising mutant cells marked as # expressed Gsh2. (C) Rostrally, Gsh2 expression was observed in *Gli3*$^{-/-}$ cells in dorsal telencephalon (boxed area shown in F). Abbreviations: Vtel, ventral telencephalon; others as in Figs. 1–4. Scale bars: A–C=400 μm; D–I=50 μm.

widely expressed in the dorsal telencephalon of *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeras, including *Gli3*$^{-/-}$ clusters overlying the neocortex (marked as * in Fig. 7A). It was excluded from the segregated clusters of *Gli3*$^{-/-}$ telencephalic cells situated dorsomedially in the telencephalon (boxed area marked with * in Fig. 7A, shown in E; Fig. 9D), consistent with these cells having an identity associated with thalamic cells (E12.5, $n=2$; E14.5, $n=3$; E15.5, $n=2$). The region designated # showed a pattern of Tbr2 expression similar to than observed in the eminentia thalami (Fig. 7A,C). Clusters of *Gli3*$^{-/-}$ cells on the dorsal side of the PSPB were negative for Tbr2, which is not expressed by ventral telencephalic cells (Figs. 8C,D and 9B).

Pax6 expression

Normally, Pax6 is expressed by cells in the dorsal telencephalon, the LGE, the eminentia thalami and the ventral thalamus (Walther and Gruss, 1991). In *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeras (E14.5, $n=3$), Pax6 was expressed in cells of both genotypes in the dorsal telencephalon, including clusters of *Gli3*$^{-/-}$ neocortical cells separated from the ventricular zone (e.g. arrowheads in Fig. 7B), in the eminentia thalami and, in a similar pattern, in the region designated "#" (Figs. 7B,D).

Nkx2.2 expression

The transcription factor Nkx2.2 is expressed in a highly restricted domain in the diencephalon, immediately anterior to the zona limitans intrathalamica (ZLI), which delimits the dorsal thalamus from the ventral thalamus (Fig. 7F; Kitamura et al., 1997; Price, 1993; Vue et al., 2007). We did not observe expression of Nkx2.2 in any area of the telencephalon in *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeras (E14.5, $n=2$) (Fig. 7F), suggesting that telencephalic *Gli3*$^{-/-}$ cells do not acquire a diencephalic identity associated with ventral thalamic cells close to the ZLI.

Mash1 expression

Normally, Mash1 is expressed only on the ventral side of the PSPB (Casarosa et al., 1999), but it was found extending into segregated clusters of *Gli3*$^{-/-}$ cells on the dorsal side of the PSPB in *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeras (E12.5, $n=1$; E14.5, $n=2$; E15.5, $n=1$) (Figs. 8A,B). At rostral levels in *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeras there was ectopic expression of Mash1 in clusters of *Gli3*$^{-/-}$ cells located dorsal to the PSPB (Fig. 8E) that did not express Pax6 (Fig. 8F). These data are compatible with the hypothesis that *Gli3*$^{-/-}$ cells in the lateral part of the dorsal telencephalon of experimental chimeras express transcription factors characteristic of ventral telencephalon.

Gad67 expression

The synthesis of GABA in the brain depends on the enzyme glutamate decarboxylase (GAD) and most GABAergic cells express the isoform Gad67. Most GABAergic interneurons in the cerebral cortex originate in ventral telencephalon and migrate into dorsal telencephalon from about E13 onwards. At first, most take up positions in the marginal, lower intermediate, subventricular and ventricular zones until postnatal ages, when they occupy the layers of the developing cortical plate (López-Bendito et al., 2004; Wonders and Anderson, 2006). We compared the expression of Gad67 in *Gli3*$^{-/-}$ and *Gli3*$^{+/+}$ cells in the dorsal telencephalon at E14.5; Gad67 expression was

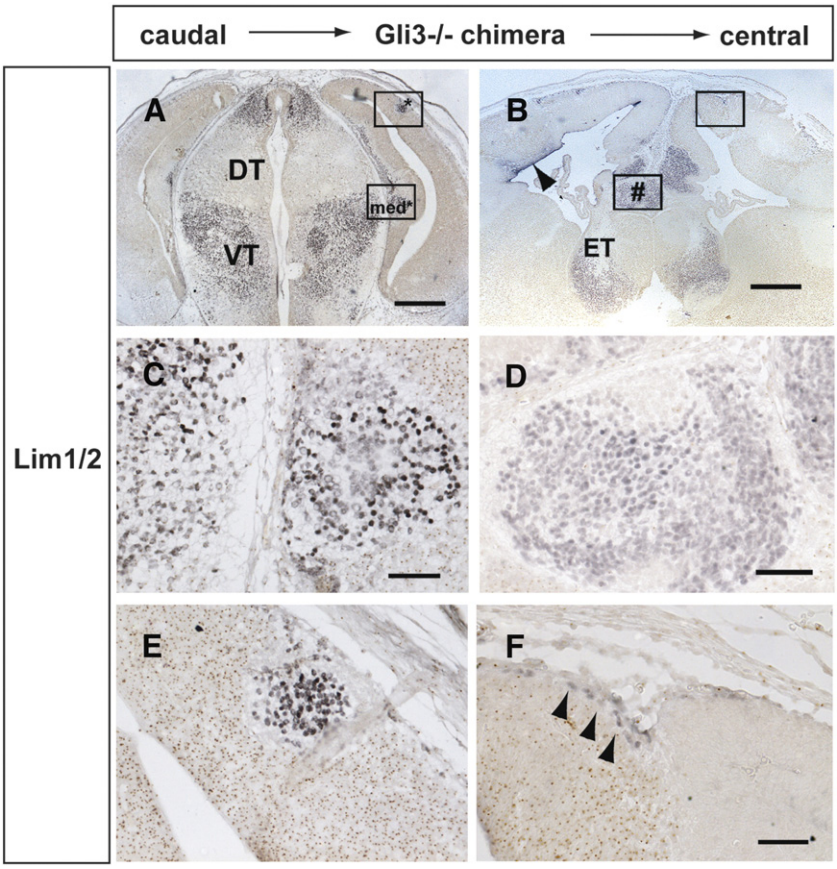

Fig. 6. *Gli3*$^{-/-}$ mutant cells in medial location in the telencephalon of *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeras express Lim1/2. Immunohistochemistry on E14.5 *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimera in which wild type cells are *Tg*-positive. (A) In caudal sections, Lim1/2 is expressed in the ventral thalamus and also in medially located telencephalic clusters of *Gli3*$^{-/-}$ cells (boxed areas shown in C,E). (B) Further rostrally, both eminentia thalami and the *Gli3*$^{-/-}$ tissue adjacent to it (designated #) express Lim1/2 (boxed area shown in D). Lim1/2 is also expressed in Cajal Retzius cells (arrowheads in F) in the cortical plate of the developing dorsal telencephalon but neither in mutant nor in wild type cells through the rest of the depth of the neocortex (boxed area in F). Arrowhead in B indicates a staining artefact. Abbreviations: as in Figs. 1–4. Scale bars: A,B=400 μm; C–F=50 μm.

widespread in cells of both genotypes in the ventral telencephalon, as it is in wild type embryos at this age. Figs. 9A,C shows that clusters of *Gli3*$^{-/-}$ cells situated in the cortical plate of the lateral (Fig. 9A) or medial (Fig. 9C) parts of the dorsal telencephalon expressed Gad67, whereas most of the surrounding wild type cortical plate cells did not. Figs. 9B,D show *Tg*-positive *Gli3*$^{-/-}$ cells, that are Tbr2-negative (as described above), in adjacent sections to those in Figs. 9A,B. In contrast to these findings for *Gli3*$^{-/-}$ cells close to the dorsal telencephalic boundaries, most *Gli3*$^{-/-}$ cells in central regions of the dorsal telencephalon did not show increased expression of Gad67 (not shown).

Dlx2 expression

We observed that clusters of *Gli3*$^{-/-}$ cells situated in lateral and medial, but not central, parts of the chimeric dorsal telencephalon expressed the normally ventrally restricted transcription factor Dlx2 (E12.5, $n=1$; E14.5, $n=2$; E15.5, $n=1$) (Bulfone et al., 1993). An example of a laterally located cluster of *Gli3*$^{-/-}$ cells immunopositive for Dlx2 is shown in Fig. 9E.

Nkx2.1 expression

Nkx2.1 expression is normally restricted to the MGE and other ventral structures including the septum and preoptic area (Sussel et al., 1999). This pattern of expression was maintained in chimeric forebrain: Fig. 9F shows expression of Nkx2.1 in *Gli3*$^{+/+}$ and *Gli3*$^{-/-}$ (*Tg*-positive) cells in E14.5 chimeric ventral forebrain. Neither *Gli3*$^{-/-}$ (*Tg*-positive) nor wild type cells in dorsal telencephalon were positive for Nkx2.1 (Fig. 9G).

Discussion

Our main findings are: (1) *Gli3*$^{-/-}$ cells are present in all components of the *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeric forebrain in proportions that either equal or exceed the proportions found elsewhere in the embryo; (2) *Gli3*$^{-/-}$ cells segregate from *Gli3*$^{+/+}$ cells forming many abnormal structures particularly in dorsal telencephalon and close to the boundary of telencephalon with diencephalon; (3) *Gli3*$^{-/-}$ cells in the *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ telencephalon express abnormal sets of molecular markers that vary with location (summarized in Fig. 10A).

Gli3$^{-/-}$ *cells are present in all forebrain tissue in chimeras*

Many studies of *Gli3*$^{-/-}$ mutants have indicated that developmental distortions of brain structures are caused by selective expansion of some tissues with reduction or absence of others (e.g. (Aoto et al., 2002; Blaess et al., 2008; Fotaki et al., 2006; Franz, 1994; Franz and Besecke, 1991; Grove et al., 1998; Hui and Joyner, 1993; Johnson, 1967; Theil et al., 1999; Tole et al., 2000; Zaki et al., 2005). In the forebrain of *Gli3*$^{-/-}$ embryos, the cerebral cortex is reduced in size with complete absence of dorsomedial telencephalon whereas the ventral telencephalon and diencephalon appear enlarged. Here we found that, in chimeras, *Gli3*$^{-/-}$ cells contribute to all forebrain tissues, including those that are missing in *Gli3*$^{-/-}$ mutants, in proportions that either exceed or are similar to the proportions contributing elsewhere in the embryo. The excessive contributions of mutant cells in some tissues might be explained if the absence of Gli3 from these cells enhances their ability to proliferate and/or survive. Our analysis of densities of

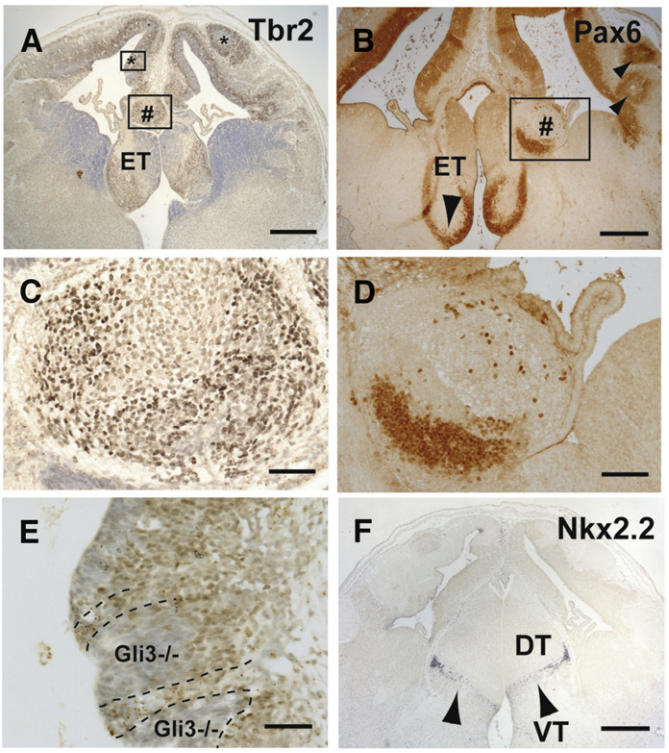

Fig. 7. Altered expression of Tbr2 and Pax6 in *Gli3*$^{-/-}$ cells in dorsal telencephalon of *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimeras. Immunohistochemistry on E14.5 *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimera in which wild type cells are *Tg*-positive. (A) Tbr2 is expressed in the dorsal telencephalon, in the tissue marked # (boxed area shown in C) and in the eminentia thalami but not in medial clusters of *Gli3*$^{-/-}$ cells (boxed area shown in E). (B) Pax6 is expressed in the tissue marked # (boxed area shown in D) and in the eminentia thalami (arrowhead in B). (F) At E14.5, Nkx2.2 is expressed at the zona limitans intrathalamica (arrowheads) of the diencephalon and remains restricted to this domain in the E14.5 *Gli3*$^{-/-}$↔*Gli3*$^{+/+}$ chimera. Abbreviations: as in Figs. 1–4. Scale bars: A, B, F=400 μm; C–E=50 μm.

dividing (pH3-expressing) cells in neocortical *Gli3*$^{-/-}$ clusters suggests that proliferation of *Gli3*$^{-/-}$ cells might be enhanced. Lack of Gli3 has been shown to enhance proliferation and also attenuate cell death elsewhere in the developing brain, in the midbrain and hindbrain (Blaess et al., 2008). Previous *in vitro* work has shown that loss of Gli3 from embryonic telencephalic cells attenuates their death, although in this *in vitro* context proliferation was not enhanced (Zaki et al., 2005). A complete understanding of proliferation and survival rates of *Gli3*$^{-/-}$ cells in chimeras will require more study in the future.

A major conclusion of our current work is that Gli3 is not required autonomously for cells to exist within the dorsomedial telencephalon, a region that does not exist in *Gli3*$^{-/-}$ embryos. When chimeric analysis was used to examine the functions of another transcription factor, Pax6, in eye development, it was found that there was no contribution of *Pax6*$^{-/-}$ cells to eye structures that are completely absent in *Pax6*$^{-/-}$ embryos, suggesting that Pax6 is required autonomously by cells of the missing structures for their generation and/or survival (Quinn et al., 2007). In contrast to that situation, we conclude that Gli3 is not required to generate persistent populations of cells situated in the dorsomedial telencephalon; our results argue against the possibility that this tissue is absent in *Gli3*$^{-/-}$ mutants because *Gli3*$^{-/-}$ cells have an intrinsic inability to proliferate and survive in this location. Rather, our results suggest that *Gli3*$^{-/-}$ cells in this location have an intrinsic inability to adopt a dorsomedial telencephalic identity.

Gli3$^{-/-}$ *cells segregate from Gli3*$^{+/+}$ *cells and express abnormal sets of molecular markers that vary with location*

In all regions of the forebrain of chimeras *Gli3*$^{-/-}$ cells segregated from *Gli3*$^{+/+}$ cells, with the degree of segregation most extreme in dorsal telencephalon where it led to the generation of highly abnormal *Gli3*$^{-/-}$ structures. This suggests that in dorsal telencephalon *Gli3*$^{-/-}$ and *Gli3*$^{+/+}$ cells express very different sets of molecules regulating their cell-surface properties, whereas in ventral telencephalon and diencephalon differences are fewer. The properties of cells are controlled by the cocktails of transcription factors that they express and so we hypothesized that the extreme segregation of mutant and wild-type cells in dorsal telencephalon might arise from major differences in their identities, in terms of their expression of key transcription factors. Our analysis demonstrated that such differences exist.

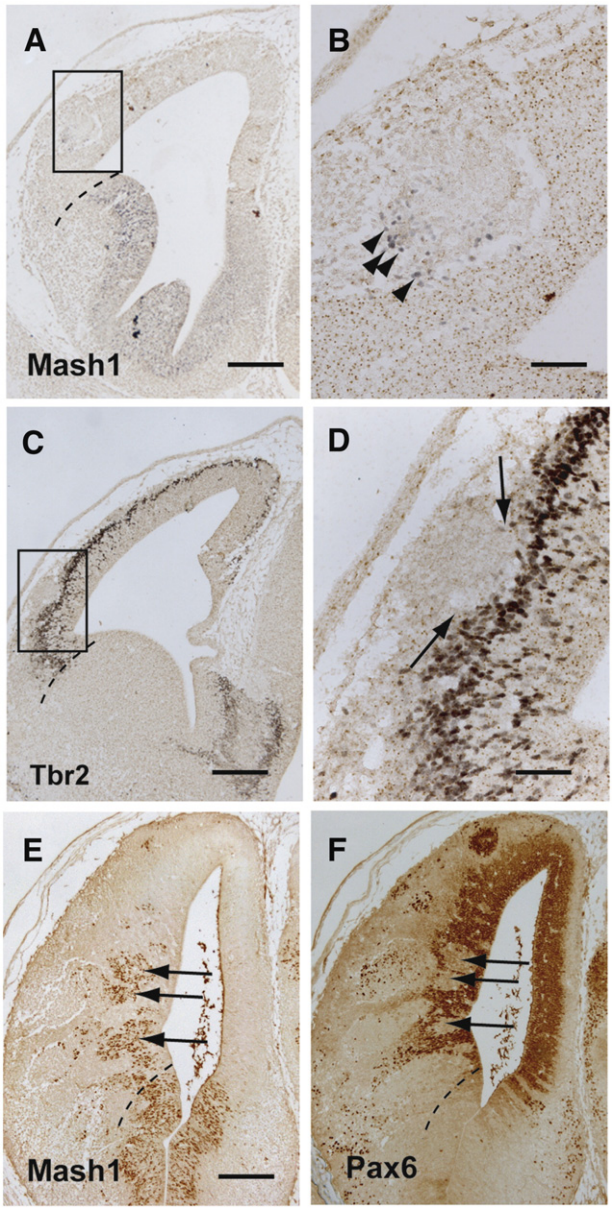

Fig. 8. *Gli3*$^{-/-}$ cells in lateral and rostral dorsal telencephalon of chimeras exhibit ventral telencephalic identities. (A, B) Section through an E12.5 chimera showing a cluster of *Tg*-negative *Gli3*$^{-/-}$ cells in lateral dorsal telencephalon (PSPB is marked with a broken line in A) which ectopically express the ventral telencephalic marker Mash1 (boxed area shown in B). (C, D) Section through an E12.5 chimera: clusters of *Tg*-negative *Gli3*$^{-/-}$ cells in lateral dorsal telencephalon do not express the dorsal telencephalic marker Tbr2 (boxed area is shown in D; *Gli3*$^{-/-}$ cells are between the arrows). (E, F) Adjacent sections of rostral telencephalon of an E14.5 chimera, showing Mash1-positive, Pax6-negative clusters of *Gli3*$^{-/-}$ cells located dorsal to the PSPB (broken line) in the rostral telencephalon. Arrows point to examples of *Gli3*$^{-/-}$ cell clusters. Scale bars: A, C, E, F=200 μm; B, D=50 μm.

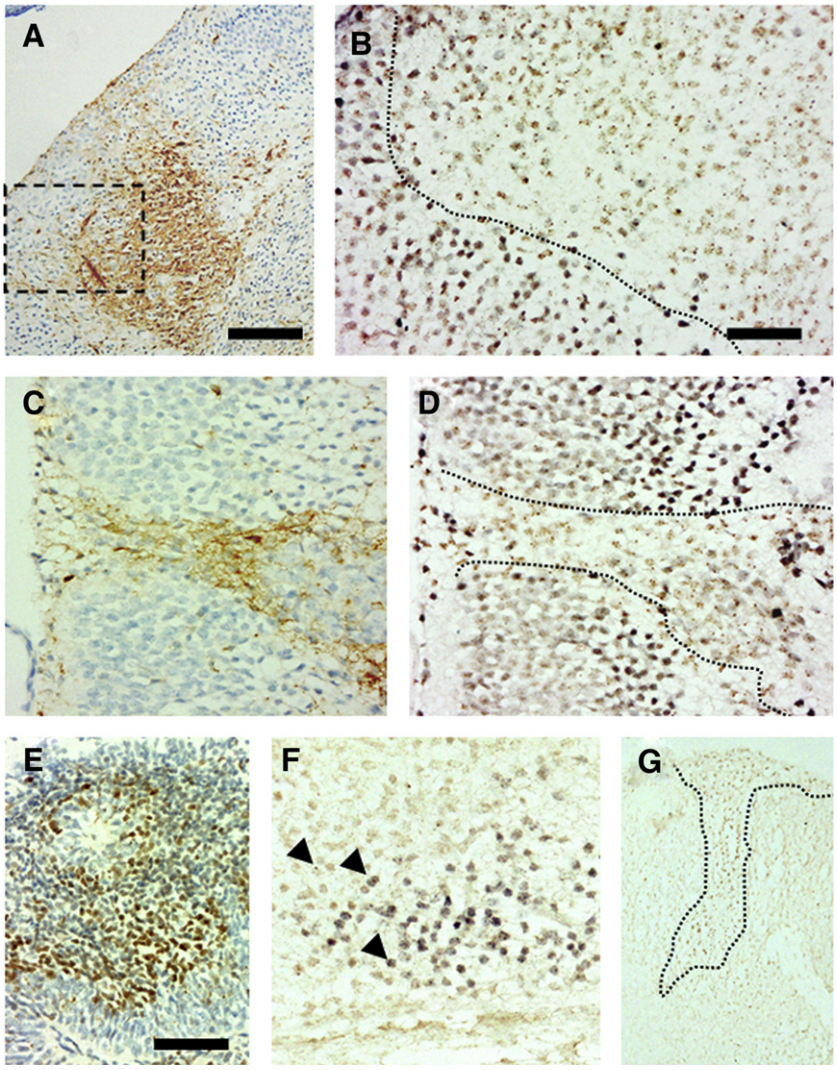

Fig. 9. *Gli3*$^{-/-}$ cells in dorsal telencephalon of chimeras express Gad67 and Dlx2 but not Nkx2.1. Sections of an E14.5 chimera: (A, C) two clusters of Gad67-positive *Gli3*$^{-/-}$ *Tg*-positive cells. (A) This cluster is situated in the lateral part of the cortex (Gad67 immunoreactivity is brown). (B) A section adjacent to that in A, showing the area outlined in A: *Gli3*$^{-/-}$ *Tg*-positive cells are to the right of the dotted line and contain a brown dot in their nuclei. The section is also immunostained for Tbr2, which is expressed by the wild type cells to the left of the broken line and not by the mutant cells. (C) This cluster is situated in the dorsomedial part of the cortex. (D) An adjacent section confirms that the Gad67-positive cluster comprises *Tg*-positive Tbr2-negative cells. (E) A cluster of *Gli3*$^{-/-}$ mutant cells in the lateral cortex of an E14.5 chimera containing Dlx2-positive cells. (F) Cells in the ventral forebrain of an E14.5 chimera are positive for Nkx2.1: some cells are *Gli3*$^{-/-}$ (*Tg*-positive: arrowheads). (G) A cluster of *Gli3*$^{-/-}$ *Tg*-positive cells in the lateral cortex of the same chimera as in F, double-stained for Nkx2.1: no expression of Nkx2.1 was detected. Scale bars: A,G, 400 μm; E, 200 μm; B–D, F, 100 μm.

In parts of the dorsal telencephalon near to its boundary with the diencephalon, mutant cells expressed sets of transcription factors normally expressed by cells only on the diencephalic side of the boundary (Gsh2, Lim1/2, Dlx2, but not Foxg1; Fig. 10A). Particularly striking was the generation of a structure designated # in Results that appeared, on the basis of its organization and pattern of gene expression, to be a duplication of the eminentia thalami at the telencephalic/diencephalic border (Lim1/2-positive, Tbr2-positive, Pax6-positive, but Gsh2-negative and Foxg1-negative: Fig. 10A). In rostral and lateral parts of the dorsal telencephalon near to the ventral telencephalon, mutant cells also expressed sets of transcription factors normally expressed by cells only on the ventral side of the boundary (Gsh2, Mash1 and Dlx2; Fig. 10A). Mutant cells in dorsal telencephalon expressed neither Nkx2.1 nor Nkx2.2; these markers are normally expressed by cells at a distance from the PSPB, in the MGE, and at a distance from the diencephalic/telencephalic border, immediately anterior to the ZLI (Price, 1993; Kitamura et al., 1997; Sussel et al., 1999; Vue et al., 2007).These findings suggest that mutant cells in the dorsal telencephalon that are near to its boundaries express transcription factors normally restricted to cells in immediately adjacent territory. Furthermore, we observed that these dorsal telencephalic cells express abnormally high levels of Gad67, which is expressed by GABAergic interneurons. A number of studies have implicated molecules including Dlx2, Mash1 and Shh in the specification of GABAergic interneurons in the ventral telencephalon (reviewed by Wonders and Anderson, 2006) and the ectopic expression of Dlx2 and Mash1 in *Gli3*$^{-/-}$ dorsal telencephalic cells is a possible explanation for the high levels of Gad67 expression by these cells.

A model that might explain these findings is illustrated in Figs. 10B, C. It is known that diffusible morphogens such as bone morphogenetic factors (Bmps), fibroblast growth factors (Fgfs), Shh and Wnts released by cells within and around the developing neural plate pattern its major regions, regulating their molecular identities and subsequent development by causing the activation or repression of specific sets of transcription factors (for recent reviews see Aboitiz and Montiel, 2007; Hebert and Fishell, 2008; Takahashi and Liu, 2006). Patterning also depends on the way in which cells in each region respond to the morphogens in their environment; this property, referred to as developmental competence, varies regionally. While diffusible

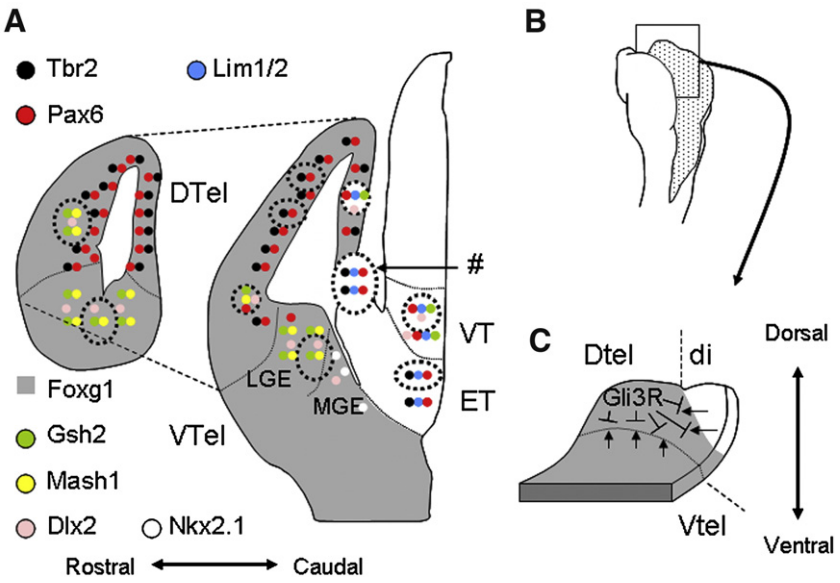

Fig. 10. Summary of the identity changes of *Gli3*$^{-/-}$ cells in the dorsal telencephalon of chimeras. (A) Diagram illustrates the left side of the brain. Groups of *Gli3*$^{-/-}$ cells are outlined with dotted lines: those situated medially in the dorsal telencephalon adopt the identities of diencephalic cells, while those situated laterally in the dorsal telencephalon adopt the identities of ventral telencephalic cells. (B) Dorsal view of the head folds of the rostral neural plate and (C) model suggesting a possible action of Gli3R, which is highly expressed in dorsal telencephalon where it might prevent the actions of morphogens that would otherwise convert dorsal telencephalic cells to ventral telencephalic and diencephalic identities. Abbreviations: as in previous Figs.

morphogens that normally specify competent diencephalic and ventral telencephalic cells might cross the boundaries into dorsal telencephalon, dorsal telencephalic cells might be incompetent to respond to those morphogens. We suggest that an important action of Gli3 in dorsal telencephalon might be to prevent its cells responding to morphogens that pattern the diencephalon and ventral telencephalon (Fig. 10C). The expression of Gli3 is compatible with this suggestion, being high in dorsal telencephalon, where posttranslational modification of its product generates high levels of a short repressor form (Gli3R), and declining through ventral telencephalon and diencephalon (Fotaki et al., 2006). This model would explain why dorsal telencephalic cells lacking Gli3 adopt the identities of diencephalic and ventral telencephalic cells only if they are relatively close to the dorsal telencephalic boundaries. What the relevant morphogens might be is currently unclear; Wnts, Bmps and Fgfs are good candidates since they are expressed in these regions (Grove et al., 1998; Shimogori et al., 2004; Theil et al., 2002) and Gli3R can inhibit responses to Wnts by antagonizing active β-catenin (Ulloa and Briscoe, 2007).

Although *Gli3*$^{-/-}$ dorsal telencephalic cells in a central part of the neocortex, i.e. distant from its lateral, rostral and medial boundaries, showed no alterations in their expression of the set of markers analysed here, they did show extreme segregation from their wild-type neighbours. The fact that they segregate indicates that there are molecular differences but further work is needed to discover their nature. The number of possible cell-surface molecular changes that might account for segregation of *Gli3*$^{-/-}$ cells in the chimeras is large, and might include changes in the expression of members of the cell adhesion molecule, cadherin and integrin families. For example, R-cadherin is expressed in dorsal telencephalon with a boundary of expression at the PSPB (Stoykova et al., 1997); it is possible that loss of this adhesion molecule in *Gli3*$^{-/-}$ cells in dorsal telencephalon might contribute to their segregation.

Conclusion

The aetiology of the numerous defects in the forebrain of *Gli3*$^{-/-}$ mutants is likely to be complex, involving defects in the production and response to signalling molecules that pattern this region early in its development. Previous work concentrated on a consideration of the consequences of the known loss of signalling molecules as a result of the failure of formation of key dorsomedial telencephalic signalling centres (Fotaki et al., 2006; Kuschel et al., 2003; Rallu et al., 2002; Theil et al., 1999; Tole et al., 2000; Vyas et al., 2003). Here, we have used chimeras to dissect the other side of the process, namely the cell autonomous actions of Gli3 in forebrain development. We suggest that a major cell autonomous action of Gli3 is to prevent dorsal telencephalic cells adopting identities inappropriate to this region.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.12.008.

References

Aboitiz, F., Montiel, J., 2007. Co-option of signaling mechanisms from neural induction to telencephalic patterning. Rev. Neurosci. 18, 311–342.
Aoto, K., Nishimura, T., Eto, K., Motoyama, J., 2002. Mouse GLI3 regulates Fgf8 expression and apoptosis in the developing neural tube, face, and limb bud. Dev. Biol. 251, 320–332.
Blaess, S., Corrales, J.D., Joyner, A.L., 2006. Sonic hedgehog regulates Gli activator and repressor functions with spatial and temporal precision in the mid/hindbrain region. Development 133, 1799–1809.
Blaess, S., Stephen, D., Joyner, A.L., 2008. Gli3 coordinates three-dimensional patterning and growth of the tectum and cerebellum by integrating Shh and Fgf8 signaling. Development 135, 2093–2103.
Brox, A., Puelles, L., Ferreiro, B., Medina, L., 2004. Expression of the genes Emx1, Tbr1, and Eomes (Tbr2) in the telencephalon of *Xenopus laevis* confirms the existence of a ventral pallial division in all tetrapods. J. Comp. Neurol. 474, 562–577.
Bulfone, A., Puelles, L., Porteus, M.H., Frohman, M.A., Martin, G.R., Rubenstein, J.L., 1993. Spatially restricted expression of Dlx-1, Dlx-2 (Tes-1), Gbx-2, and Wnt-3 in the embryonic day 12.5 mouse forebrain defines potential transverse and longitudinal segmental boundaries. J. Neurosci. 13, 3155–3172.

Casarosa, S., Fode, C., Guillemot, F., 1999. Mash1 regulates neurogenesis in the ventral telencephalon. Development 126, 525–534.
Copp, A.J., 1994. Genetic models of mammalian neural tube defects. Ciba Found. Symp. 181, 118–134 discussion 134–43.
Englund, C., Fink, A., Lau, C., Pham, D., Daza, R.A., Bulfone, A., Kowalczyk, T., Hevner, R.F., 2005. Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. J. Neurosci. 25, 247–251.
Fotaki, V., Yu, T., Zaki, P.A., Mason, J.O., Price, D.J., 2006. Abnormal positioning of diencephalic cell types in neocortical tissue in the dorsal telencephalon of mice lacking functional Gli3. J. Neurosci. 26, 9282–9292.
Franz, T., 1994. Extra-toes (Xt) homozygous mutant mice demonstrate a role for the Gli-3 gene in the development of the forebrain. Acta Anat. (Basel) 150, 38–44.
Franz, T., Besecke, A., 1991. The development of the eye in homozygotes of the mouse mutant Extra-toes. Anat. Embryol. (Berl) 184, 355–361.
Grove, E.A., Tole, S., Limon, J., Yip, L., Ragsdale, C.W., 1998. The hem of the embryonic cerebral cortex is defined by the expression of multiple Wnt genes and is compromised in Gli3-deficient mice. Development 125, 2315–2325.
Hebert, J.M., Fishell, G., 2008. The genetics of early telencephalon patterning: some assembly required. Nat. Rev. Neurosci. 9, 678–685.
Hsieh-Li, H.M., Witte, D.P., Szucsik, J.C., Weinstein, M., Li, H., Potter, S.S., 1995. Gsh-2, a murine homeobox gene expressed in the developing brain. Mech. Dev. 50, 177–186.
Hui, C.C., Joyner, A.L., 1993. A mouse model of greig cephalopolysyndactyly syndrome: the extra-toesJ mutation contains an intragenic deletion of the Gli3 gene. Nat. Genet. 3, 241–246.
Johnson, D.R., 1967. Extra-toes: anew mutant gene causing multiple abnormalities in the mouse. J. Embryol. Exp. Morphol. 17, 543–581.
Kitamura, K., Miura, H., Yanazawa, M., Miyashita, T., Kato, K., 1997. Expression patterns of Brx1 (Rieg gene), Sonic hedgehog, Nkx2.2, Dlx1 and Arx during zona limitans intrathalamica and embryonic ventral lateral geniculate nuclear formation. Mech. Dev. 67, 83–96.
Kuschel, S., Ruther, U., Theil, T., 2003. A disrupted balance between Bmp/Wnt and Fgf signaling underlies the ventralization of the Gli3 mutant telencephalon. Dev. Biol. 260, 484–495.
Lo, C.W., Coulling, M., Kirby, C., 1987. Tracking of mouse cell lineage using microinjected DNA sequences: analyses using genomic Southern blotting and tissue-section in situ hybridizations. Differentiation 35, 37–44.
López-Bendito, G., Sturgess, K., Erdélyi, F., Szabó, G., Molnár, Z., Paulsen, O., 2004. Preferential origin and layer destination of GAD65-GFP cortical interneurons. Cereb. Cortex 14, 1122–1133.
Maynard, T.M., Jain, M.D., Balmer, C.W., LaMantia, A.S., 2002. High-resolution mapping of the Gli3 mutation extra-toes reveals a 51.5-kb deletion. Mamm. Genome 13, 58–61.
Meyer, N.P., Roelink, H., 2003. The amino-terminal region of Gli3 antagonizes the Shh response and acts in dorsoventral fate specification in the developing spinal cord. Dev. Biol. 257, 343–355.
Price, M., 1993. Members of the Dlx- and Nkx2-gene families are regionally expressed in the developing forebrain. J. Neurobiol. 24, 1385–1399.
Quinn, J.C., Molinek, M., Martynoga, B.S., Zaki, P.A., Faedo, A., Bulfone, A., Hevner, R.F., West, J.D., Price, D.J., 2007. Pax6 controls cerebral cortical cell number by regulating exit from the cell cycle and specifies cortical cell identity by a cell autonomous mechanism. Dev. Biol. 302, 50–65.
Rallu, M., Machold, R., Gaiano, N., Corbin, J.G., McMahon, A.P., Fishell, G., 2002. Dorsoventral patterning is established in the telencephalon of mutants lacking both Gli3 and Hedgehog signaling. Development 129, 4963–4974.
Rash, B.G., Grove, E.A., 2007. Patterning the dorsal telencephalon: a role for sonic hedgehog? J. Neurosci. 27, 11595–11603.
Sheng, H.Z., Bertuzzi, S., Chiang, C., Shawlot, W., Taira, M., Dawid, I., Westphal, H., 1997. Expression of murine Lhx5 suggests a role in specifying the forebrain. Dev. Dyn. 208, 266–277.
Shimogori, T., Banuchi, V., Ng, H.Y., Strauss, J.B., Grove, E.A., 2004. Embryonic signaling centers expressing BMP, WNT and FGF proteins interact to pattern the cerebral cortex. Development 131, 5639–5647.
Stoykova, A., Götz, M., Gruss, P., Price, J., 1997. Pax6-dependent regulation of adhesive patterning, R-cadherin expression and boundary formation in developing forebrain. Development 124, 3765–3777.
Sussel, L., Marin, O., Kimura, S., Rubenstein, J.L., 1999. Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. Development 126, 3359–3370.
Takahashi, H., Liu, F.C., 2006. Genetic patterning of the mammalian telencephalon by morphogenetic molecules and transcription factors. Birth Defects Res. C Embryo Today 78, 256–266.
Tam, P.P.L., Rossant, J., 2003. Mouse embryonic chimeras: tools for studying mammalian development. Development 130, 6155–6163.
Tao, W., Lai, E., 1992. Telencephalon-restricted expression of BF-1, a new member of the HNF-3/fork head gene family, in the developing rat brain. Neuron 8, 957–966.
Theil, T., 2005. Gli3 is required for the specification and differentiation of preplate neurons. Dev. Biol. 286, 559–571.
Theil, T., Alvarez-Bolado, G., Walter, A., Ruther, U., 1999. Gli3 is required for Emx gene expression during dorsal telencephalon development. Development 126, 3561–3571.
Theil, T., Aydin, S., Koch, S., Grotewold, L., Ruther, U., 2002. Wnt and Bmp signalling cooperatively regulate graded Emx2 expression in the dorsal telencephalon. Development 129, 3045–3054.
Tole, S., Ragsdale, C.W., Grove, E.A., 2000. Dorsoventral patterning of the telencephalon is disrupted in the mouse mutant extra-toes(J). Dev. Biol. 217, 254–265.
Turksen, K., 2002. Embryonic Stem Cells, Methods and Protocols. Humana Press, Totowa, New Jersey.
Ulloa, F., Briscoe, J., 2007. Morphogens and the control of cell proliferation and patterning in the spinal cord. Cell Cycle 6, 2640–2649.
Vortkamp, A., Franz, T., Gessler, M., Grzeschik, K.H., 1992. Deletion of GLI3 supports the homology of the human Greig cephalopolysyndactyly syndrome (GCPS) and the mouse mutant extra toes (Xt). Mamm. Genome 3, 461–463.
Vue, T.Y., Aaker, J., Taniguchi, A., Kazemzadeh, C., Skidmore, J.M., Martin, D.M., Martin, J.F., Treier, M., Nakagawa, Y., 2007. Characterization of progenitor domains in the developing mouse thalamus. J. Comp. Neurol. 505, 73–91.
Vyas, A., Saha, B., Lai, E., Tole, S., 2003. Paleocortex is specified in mice in which dorsal telencephalic patterning is severely disrupted. J. Comp. Neurol. 466, 545–553.
Walther, C., Gruss, P., 1991. Pax-6, a murine paired box gene, is expressed in the developing CNS. Development 113, 1435–1449.
West, J.D., Flockhart, J.H., 1994. Genotypically unbalanced diploid < = = > diploid foetal mouse chimaeras: possible relevance to human confined mosaicism. Genet. Res. 63, 87–99.
Winter, R.M., Huson, S.M., 1988. Greig cephalopolysyndactyly syndrome: a possible mouse homologue (Xt-extra toes). Am. J. Med. Genet. 31, 793–798.
Wonders, C.P., Anderson, S.A., 2006. The origin and specification of cortical interneurons. Nat. Rev., Neurosci. 7, 687–696.
Xuan, S., Baptista, C.A., Balas, G., Tao, W., Soares, V.C., Lai, E., 1995. Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres. Neuron 14, 1141–1152.
Yamazaki, H., Sekiguchi, M., Takamatsu, M., Tanabe, Y., Nakanishi, S., 2004. Distinct ontogenic and regional expressions of newly identified Cajal–Retzius cell-specific genes during neocorticogenesis. Proc. Natl. Acad. Sci. U. S. A. 101, 14509–14514.
Zaki, P.A., Martynoga, B., Delafield-Butt, J.T., Fotaki, V., Yu, T., Price, D.J., 2005. Loss of Gli3 enhances the viability of embryonic telencephalic cells in vitro. Eur. J. Neurosci. 22, 1547–1551.
Zaki, P.A., Collinson, J.M., Toraiwa, J., Simpson, T.I., Price, D.J., Quinn, J.C., 2006. Penetrance of eye defects in mice heterozygous for mutation of Gli3 is enhanced by heterozygous mutation of Pax6. BMC Dev. Biol. 6, 46.